Interleukin-37b inhibits the growth of murine endometriosis-like lesions by regulating proliferation, invasion, angiogenesis and inflammation

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ABSTRACT: Endometriosis is a gynecological disease with abnormal expression of interleukin (IL)-37 which can suppress inflammation and the immune system. Here we investigated the role of the IL-37b splice variant in endometriosis in vivo and in vitro. In a murine model of endometriosis, in vivo administration of IL-37b significantly inhibited the development of lesions judged by the number (P = 0.0213), size (P = 0.0130) and weight (P = 0.0152) of lesions. IL-37b had no effect on the early stage of lesion formation, however administration in the growth stage of lesions decreased the number (P = 0.0158), size (P = 0.0158) and weight (P = 0.0258) of lesions compared with PBS control, an effect that was not reversed by macrophage depletion. Expressions of inflammatory factors, matrix metalloproteinases and vascular endothelial growth factor-A mRNA/protein were significantly inhibited in ectopic lesions following IL-37b administration, and in uterine segments treated in vitro. In vitro treatment of uterine segments with IL-37b inhibited phosphorylation of Akt and Erk1/2 in uterine segments. Isolated mouse endometrial stromal treated with IL-37b and transfected with pIL-37b plasmid got suppressed cell proliferation, invasion, angiogenesis and the expression of inflammatory factors. In addition, transfection with pIL-37b significantly decreased the phosphorylation of Akt and Erk1/2. IL-37b also inhibited proliferation and the expression of inflammatory and angiogenesis factors in epithelial cell line RL95–2. These findings suggest that IL-37b may inhibit the growth of lesions by regulating proliferation, invasion, angiogenesis and inflammation through Akt and Erk1/2 signaling pathway.

Key words: endometriosis / interleukin-37b / uterine tissue segments / endometrial cells / macrophages

Introduction

Endometriosis is a chronic gynecological disease characterized by presence of endometrial glands and stroma outside uterine cavity, which affects 6–10% of women in reproductive age (Giudice and Kao, 2004) and mainly results in chronic pain and infertility (Crosignani et al., 2006). Despite many existing theories, the pathogenesis of endometriosis is still not fully understood. Inflammation and immune dysfunction have been proposed to be crucial for the development of endometriosis (Santulli et al., 2015; Symons et al., 2018; Zhou et al., 2019). Studies have identified a range of inflammatory factors, including interleukin (IL)-1β, IL-6, IL-8, IL-10, tumor necrosis factor (TNF)-α and transforming growth factor (TGF)-β to be expressed abnormally in endometriosis patients (Kyama et al., 2008; Li et al., 2017a; Overton et al., 1996; Sikora et al., 2017; Zhou et al., 2019). Furthermore, the number of macrophages and neutrophil was elevated and the phagocytic capacity of macrophages and natural killer (NK) cells was reduced in endometriosis patients (Chuang et al., 2009; Ito et al., 2013; Jeung et al., 2016; Milewski et al., 2011).

IL-37 is a new member of IL-1 family which can suppress inflammation and immunity (Cavalli and Dinarello, 2018). IL-37 can reduce inflammation in many animal experimental models, including colitis, endotoxemia, arthritis, hepatic and renal ischemia-reperfusion (Cavalli et al., 2017; McNamee et al., 2011; Nold-Petry et al., 2015; Sakai et al., 2012;
Yang et al., 2015b). Furthermore, IL-37 can inhibit macrophage proliferation, apoptosis, migration and the expression of pro-inflammatory factors (McCurdy et al., 2017); reduce neutrophil activation and chemotaxis (Sakai et al., 2012; Wu et al., 2014); attract NK cells and enhance NK cells infiltration (Zhao et al., 2014). Considering the disorders of inflammatory factors and immune cells in endometriosis and the effect of IL-37, we speculated that IL-37 may have potential usefulness in preventing the progress of endometriosis, providing therapeutic benefits in endometriosis.

Recent studies have revealed that the expression of IL-37 in ectopic and eutopic endometrium and the level of IL-37 in blood in endometriosis patients were higher than those without endometriosis and, the expression of IL-37 correlates with the severity of endometriosis, but there remains controversy over the level of IL-37 in peritoneal fluid (Fan et al., 2018; Jiang et al., 2016; Jiang et al., 2019; Kaabachi et al., 2017). In addition, treatment with recombinant human IL-37 significantly decreased the size and weight of lesions in a suture model of endometriosis in mice, revealing that IL-37 could attenuate the progress of endometriosis (Jiang et al., 2018), but it was unknown how IL-37 affects the development of endometriosis in mice.

To further explore the role of IL-37 in endometriosis in mice, we assessed the effect of IL-37 on the development of endometriosis, peritoneal macrophage, uteri and endometrial cells. Among five different splice variants (IL-37a-e) of IL-37, IL-37β was chosen because it is the largest and most complete as it contains five of the six exons of the IL37 gene (all but exon 3) (Cavalli and Dinarello, 2018). Our results showed that IL-37β significantly inhibited the growth of ectopic lesions by inhibiting cell proliferation, invasion, angiogenesis and inflammation through Akt and Erk1/2 signaling pathway, which provide a basis for further validation and the development of a new clinical strategy for the treatment of endometriosis.

Materials and Methods

Animal model of endometriosis and treatment

Female BALB/c mice (6–8 weeks) were purchased from the laboratory animal center of Tongji Medical College (Wuhan, China). All animal experiments were performed according to the guidelines of the animal care and use committee of Tongji Medical College. All mice were kept under controlled temperature and free access to food and water. The animal model was performed as previously described (Wieser et al., 2012). All donor mice and recipient mice were injected s.c. with 100 μg/kg estradiol (Aladdin, Shanghai, China) once a week until the end of each group. Donor mice were killed 7 days following first estradiol injection and uteri were collected, dissected and cut into 1 mm segments. Then, uterine segments were injected using an 18-gauge needle into the peritoneal cavity of recipient mice. This day was defined as d0. After 3, 7 or 14 days, mice were killed by cervical dislocation, and endometriosis-like lesions from each mouse were counted, measured and weighed. The surface area of a lesion was calculated by length × width × (π/4), as previously described (Becker et al., 2008). Lesions were immediately placed into 4% paraformaldehyde for paraffin embedding or into TRIzol (ambion Invitrogen, CA, USA) for RNA extraction.

To explore the effect of IL-37b in endometriosis, we injected mice i.p. with recombinant human IL-37b/IL-1F7b protein (rhIL-37b) (R&D Systems, Minneapolis, MN, USA) (1 μg/mouse) once every other day until mice were killed. To deplete macrophages, we injected mice i.p. with Clodronate Liposomes (ClodronateLiposomes.org, Netherland) (200 μL/mouse) once every 4 days until mice were killed as described previously (Bacci et al., 2009).

Assay of gene expression by real-time PCR

Total RNA was extracted by using TRizol according to the manufacturer’s instructions, and then converted to cDNA by using ReverTra Ace, RNase Inhibitor (TOYOBO, Osaka, Japan), dNTP (Thermo Fisher Scientific, CA, USA), Oligo dT and Nuclease-Free water (Invitrogen, CA, USA). The relative quantity of the isolated mRNA was determined by real-time PCR by using SYBR® qPCR Mix (TOYOBO, Osaka, Japan) on Mx3000P Real-Time PCR System (Agilent Technologies, CA, USA) according to the manufacturer’s instructions. Expression of target mRNAs was normalized and calculated by using 2−ΔΔCt (cycle threshold) method. The sequences of the PCR primers are showed in Table I.

ELISA

Peritoneal fluid was collected by washing peritoneal cavity with 1 mL PBS, and then centrifuged at 800 g for 20 minutes at 4°C. The supernatants were collected and stored at −80°C. Levels of IL-1β, IL-6, TNF-α, IL-10, TGF-β1, matrix metalloprotease 9 (MMP9) and vascular endothelial growth factor (VEGF) in the samples were assayed using ELISA Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

Immunohistochemistry

After deparaffinization, the paraffin-embedded sections of tissues were washed three times in PBS. Next, the slides were boiled in citrate buffer (pH 6.0) for 10 minutes for antigen retrieval and then immersed in 3% hydrogen peroxide for 20 minutes to block endogenous peroxidase. After washing another three times in PBS, the slides were incubated with 5% blocking goat serum for 60 minutes at room temperature. The slides were washed three times in PBS. Next, the slides were incubated with 5% blocking goat serum for 60 minutes at room temperature. The primary antibody Anti-F4/80 antibody (1:100; Abcam, Cambridge, MA, USA), anti-CD68 antibody (1:300; Abcam, Cambridge, MA, USA), anti-pan Cytokeratin antibody (1:250; Abcam, Cambridge, MA, USA), anti-Vimentin antibody (1:400; Abcam Cambridge, MA, USA), anti-proliferating cell nuclear antigen (PCNA) (1:400; Abcam, Cambridge, MA, USA), anti-MMP9 (1:400; Abcam, Cambridge, MA, USA) and anti-VEGF (1:100; Abcam, Cambridge, MA, USA) were used, with PBS serving as the control. After three times washes with PBS, Horse-radish peroxidase (HRP)-labeled secondary antibody (1:300) was applied for 50 minutes at room temperature. Finally, slides were stained with 3,3′-diaminobenzidine (DAB) and then counterstained with hematoxylin, followed by dehydration. Images were captured by Olympus microscope, and the integrated optical density (IOD) and area of lesions were determined with Image Pro Plus 6.0 image analysis software (Media Cybernetics Inc, USA).

In vitro culture of mouse uterine tissue fragments

Uterine tissue fragments were analysed in culture using a human endometrial in-vitro model (Petitbarat et al., 2011; Rahmati et al., 2014).
In brief, normal murine uterine horns were removed, dissected longitudinally, and then cut into 2 mm pieces. Uterine fragments were placed on the top of collagen sponge gels (3–5 blocks per collagen sponge gel per well) which were immersed in medium in order to keep fragments at the interface between the medium and air. The constituents of medium were as follows: RPMI-1640 medium (HyClone, Logan, Utah, USA) supplemented with 15% fetal bovine serum, 50 nM estradiol and 50 nM progesterone (Aladdin, Shanghai, China). The uterine fragments were cultured in 12-well plates at 37°C incubator with 5% CO2. After 24 hours culture, rhIL-37b was added to the medium and change medium every day for three consecutive days. Cultured fragments were collected for RNA extraction or protein analysis by western blot.

**Western blot**

Tissues and cells were lysed with RIPA lysis buffer, PMSF and phosphatase inhibitors (Boyteomy, Shanghai, China). After centrifugation at 12,000 g for 15 minutes at 4°C, the supernatant was collected. The protein concentration in supernatant was measured by BCA protein assay kit (Boyteomy, Shanghai, China). After separation by electrophoresis on 10% sodium dodecyl sulphate—polyacrylamide gels, the proteins were transferred to BioTrace NC membrane (PALL, NY, USA). The membranes were blocked with 5% non-fat dry milk and then incubated with primary antibody at 4°C overnight. Primary antibodies were as follows: Akt (pan) (C67E7), phospho-Akt (Ser473) (193H12), anti-ERK1+ERK2 antibody [EPR17526] (1/10000, Abcam, Cambridge, MA USA) and phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (197G2) (1:1000, Cell Signaling Technology, MA, USA). After washing three times in TBST, membranes were incubated with HRP-labeled secondary antibody (1:3000) for 60 minutes at room temperature. Bands were visualized by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, USA) and analyzed by using Imagej software (National Institutes of Health, Bethesda, MD, USA).

**Isolation and culture of mouse endometrial stromal cells**

The method to isolate and culture endometrial stromal cells (ESCs) was described previously (Frolova et al., 2009). In brief, uterine horns from normal mice were collected, cleared of fatty tissues and slit longitudinally. After mincing into 1 mm pieces, the uteri were digested with 2 g/L Collagenase Type I (Sigma, St Louis, MO, USA) for 60–90 minutes at 37°C with vortexing every 15 minutes. After incubation, the cells were pooled and passed through 100 and 400 meshes. Next, the cell pellets were washed twice in PBS and added to DMEM/F12 (HyClone, Logan, Utah, USA).

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**Table 1** PCR primer sequences for mouse and human samples.

<table>
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<th>Gene</th>
<th>Genbank accession No.</th>
<th>Sense Primer (5′-3′)</th>
<th>Antisense Primer (5′-3′)</th>
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<td>Il1b</td>
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</table>

Utah, USA) containing 10% fetal calf serum at 7.5 × 10^4 cells per well in 6-well plates. ESCs treated with different concentrations of rhIL-37b for 24 hours were used for the further experiments.

For detecting ESCs markers, the cells were washed in PBS and fixed in 4% paraformaldehyde for 30 minutes. After washing, the cells were treated with 0.1% TritonX-100 for 20 minutes, and then incubated with 5% blocking goat serum for 30 minutes. Anti-pan cytokeratin antibody (1:500; Abcam, Cambridge, MA, USA), anti-vimentin antibody (1:500; Abcam, Cambridge, MA, USA) or PBS was added overnight at 4°C. After another washing, the cells were incubated with HRP-labeled secondary antibody for 30 minutes, followed by staining with DAB and hematoxylin.

Cell proliferation assay
A cell proliferation assay Cell Counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) was used according to manufacturer’s protocol. ESCs were seeded in a 96-well plate at 1 × 10^4 cells/well and cultured at 37°C for 24 hours. Then, 10 μL rhIL-37b at different concentrations was added to each well. After another 24 hours at 37°C, 10 μL CCK-8 was added to each well in the dark, incubated at 37°C for 1 hour, and the absorbance was measured at 450 nm. Data were calculated from quintuplicate samples and the wells without cells were used as the blank.

Transwell
The ESCs pre-incubated with different concentrations of rhIL-37b for 24 hours were plated in the top chamber of the transwell (Corning, Kennebunk, ME, USA) with Matrigel-coated at 5 × 10^4 cells/well in the serum-free medium, and medium containing serum was added in the lower chamber. After 24 hours at 37°C in a humidified incubator with 5% CO2, the cells that still at the top chamber were removed using a cotton swab. Methanol was used to fix the cells on the other side of the membrane, which were stained with 0.1% crystal violet. Cell numbers were counted in ten randomly chosen fields at 200× magnification.

Transfection
Plasmids pIL-37b is an expression vector carrying the cDNA encoding human IL-37b. The plasmid was constructed by the insertion of cDNA into plasmid pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) in our laboratory. Plasmids were prepared and identified as described previously (Li et al., 2017c; Luo et al., 2017). The pcDNA3.1 or pIL-37b plasmid was transfected into ESCs using Lipofectamine-2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. At 48 hours after transfection, the CCK-8 assay, transwell, real-time PCR and western blot assays were performed. For the cell proliferation assay, transfected ESCs were seeded at 2 × 10^4 cells/well for 24 hours before adding CCK-8. For the transwell assay, transfected ESCs were seeded in a Matrigel-coated transwell upper chamber at 5 × 10^4 cells/well.

Endometrial cancer cell line culture and treatment
The human endometrial cancer cell line RL95–2 was obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were maintained in DMEM/F12 medium supplied with 10% fetal calf serum and 0.005 mg/ml Insulin and cultured at 37°C in a humidified incubator with 5% CO2. RL95–2 cells treated with different concentrations of rhIL-37b for 24 hours were used to measure proliferation ability and gene expression.

Statistical analysis
All experiments were repeated three times. All data were expressed as mean value ± SEM and analyzed by Student’s t-test or one-way ANOVA. A value of P < 0.05 was considered to be statistically significant.

Results
Effect of rhIL-37b on lesion formation and growth in a murine endometriosis model
First, we confirmed that the formation of endometriosis-like lesions had been completed at 3 days after induction by animal experiment (Supplementary Fig. S1). In order to assess the effect of IL-37b on the development of endometriosis, we injected rhIL-37b or PBS i.p. (Supplementary Fig. S2A). Compared to the mice treated with PBS, the number, size and weight of endometriosis-like lesions per mouse at the mice treated with rhIL-37b were significantly decreased at 14 days after induction (Fig. 1A).

In order to further investigate the effect of IL-37b on lesion formation and growth, respectively, we treated mice with rhIL-37b at different stages (Supplementary Fig. S2B-C). At the stage of early lesion formation (d0 to d3), rhIL-37b treatment did not have a significant effect on lesions (Fig. 1B). At the stage of lesion growth (d3 to d14), rhIL-37b treatment significantly reduced the number, size and weight of lesions per mouse when compared with control (Fig. 1C), and the analysis of PCNA, which is a marker of proliferation, by immunohistochemistry has revealed that rhIL-37b treatment reduced the proliferation activity in lesions (Fig. 1D).

Endometriosis-like lesions were found in all recipient mice and mostly present on the peritoneal wall, adipose tissue and perisplenic tissue whose histological analyses confirmed that the lesions had typical endometrial glandular and stromal structures (Supplementary Fig. S3). In addition, lesions at d3 have a disturbed structure and were infiltrated with many red blood cells, while at d7 and d14, lesions had obvious epithelial and stromal cell layers plus cavity-like structures (Supplementary Fig. S3A).

Effect of rhIL-37b in the absence of macrophages on lesion formation and growth in mice
Our animal experiments have confirmed that IL-37b inhibited lesion growth, and current studies have demonstrated that immune cells play a role in lesion growth (Symons et al., 2018), so we next explored, under IL-37b treatment, the role of immune cells in vivo. Normally, macrophages are the most common leukocytes in peritoneal fluid (van Furth et al., 1979) and the number of peritoneal macrophages from endometriosis patients was higher than those from the control group (Itoh et al., 2013; Takebayashi et al., 2015). Considering that IL-37 can inhibit macrophage proliferation and migration (McCurdy et al., 2017), we focused on peritoneal macrophages.
IL-37b inhibits murine endometriotic lesion growth

Figure 1 Effect of rhIL-37b on the formation and growth of endometriosis-like lesions in mice. Endometriosis was induced by injecting uterine segments i.p. (d0) and recombinant human interleukin-37b (rhIL-37b) was administered 3 days before endometriosis induction once every 2 days (rhIL-37b group, n = 3), with PBS injection in the control (control group, n = 3). Recipient mice were killed at 3, 7 or 14 days after induction, and endometriosis-like lesions were counted, measured and weighed (A). To assess the effect of rhIL-37b on early lesion formation, PBS or rhIL-37b (n = 3) was injected i.p. at −d3, −d1 and d1 and mice were killed at d3, lesions were counted, measured and weighed (B). To assess the effect of rhIL-37b on lesion growth, PBS or rhIL-37b (n = 3) was injected i.p. at d3, d5, d7, d9, d11 and d13 and mice were killed at d14, lesions were counted, measured and weighed (C). Protein levels of the marker proliferating cell nuclear antigen (PCNA) in lesions at d14 were detected by immunohistochemistry (IHC) (Scale bar: 100 μm) and integrated optical density (IOD) and area of lesions were measured by Image Pro Plus software (D). Results are expressed as mean ± SEM from three independent animal experiments. Student’s t-test. *P < 0.05 or **P < 0.01.

Macrophages were depleted by Clodronate Liposomes (Clo) in order to assess its role in the effect of IL-37b on lesion formation and growth. The effect of macrophage depletion was confirmed by staining with the macrophage markers F4/80 and CD68 (Supplementary Fig. S4). After treatment with rhIL-37b, Clo or rhIL-37b + Clo at the stage of early endometriosis lesion formation (Fig. 2A), the number, weight and size of lesions was remarkably reduced in the Clo and rhIL-37b + Clo group when compared with control or rhIL-37b group, but there was no difference between Clo group and rhIL-37b + Clo group (Fig. 2B). After treatment with rhIL-37b or Clo or rhIL-37b + Clo at the stage of lesion growth (Fig. 2C), the number, weight and size of lesions was significantly decreased in rhIL-37b + Clo group when compared with Clo group, but there was no difference between control group and Clo group (Fig. 2D). These data showed that macrophage depletion suppressed the lesion formation and did not affect the growth of lesions. rhIL-37b treatment plus macrophage depletion suppressed not
Figure 2 Effect of rhIL-37b in the absence of macrophages on lesion formation and growth in mice. Clodronate Liposomes (Clo) were used to deplete macrophages. After receiving the injection of Clo (200 μL per mouse) at -d3 and d1, and rhIL-37b (1 μg per mouse) at -d3, −d1 and d1 (rhIL-37b + Clo group, n = 3), mice were killed at d3, compared with only receiving PBS (control group, n=3), Clo (Clo group, n = 3) or rhIL-37b (rhIL-37b group, n = 3) (A) and endometriosis-like lesions were counted, measured and weighed (B). After receiving the injection of Clo at d4, d8 and d12 and/or rhIL-37b at d4, d6, d8, d10 and d12, mice were killed at d14 (C) and endometriosis-like lesions were counted, measured and weighed (D). Results were obtained from three independent experiments and expressed as mean ± SEM. Student’s t-test. *P < 0.05 or **P < 0.01.
only lesion formation but also lesion growth, however macrophage depletion did not affect the inhibition by IL-37b of lesion growth.

**Effect of rhIL-37b on inflammatory factors, MMPs and VEGF-A in mice**

Our results have confirmed that IL-37 did not act through macrophages to affect the growth of lesions, so we turned to the lesion. Chronic inflammation is one of the characteristics of endometriosis and previous studies have revealed that inflammation could promote the development of endometriosis (Azuma et al., 2017; Lee et al., 2018), so we first explored the effect of rhIL-37b on the expression of inflammatory factors in the lesions. Real-time PCR results showed that rhIL-37b treatment decreased the mRNA expression levels of pro-inflammatory (Il1b, Il6 and Tnfa) and anti-inflammatory (Il10 and Tgfb1) factors at d14 (Fig. 3A). We also detected mRNA expression of IL-37 receptors (Il18r1 and Il1r8), which are required for IL-37 to carry out anti-inflammatory function in mice (Lunding et al., 2015; Nold-Petry et al., 2015). Our results showed that rhIL-37b treatment decreased the mRNA expression of Il18r1 and increased the mRNA expression of Sigirr, compared with control (Fig. 3A). In addition, we also detected these factors in peritoneal fluids in mice. A significant reduction in
the rhIL-37b treated group was observed for TNF-α protein when compared with control (Fig. 3B).

We also examined the expression of MMPs and VEGF-A, which play important roles in invasion and angiogenesis that is important for lesion growth. Real-time PCR results showed that the mRNA expression of Mmp2, Mmp9 and Vegfa was decreased in the rhIL-37b groups when compared with control (Fig. 3C). The protein levels of MMP9 and VEGF detected by immunohistochemistry were also reduced in ectopic lesions in the rhIL-37b treated group when compared with control (Fig. 3D). In addition, we also measured the concentrations of MMP9 and VEGF in peritoneal fluids by ELISA and found a significant reduction in MMP9 protein in the rhIL-37b treated versus the control group (Fig. 3E).

Effect of rhIL-37b on uterine segments in vitro

Our results have shown that rhIL-37b treatment decreased the expression of inflammatory factors, MMPs and VEGF in lesions in vivo, however, previous studies have demonstrated that ectopic lesions could be affected by different factors in the peritoneal cavity, including peritoneal macrophages (Loh et al., 1999), neutrophils (Takamura et al., 2016), NK cells (Itch et al., 2011) and peritoneal fluid (Cosin et al., 2010). In order to assess the direct effect of rhIL-37b on uterine segments, we cultured uterine segments in vitro. Histological results showed that uterine segments cultured in vitro for 48 hours still have the typical endometrial glandular and stromal structures (Fig. 4A). Immunohistochemistry results showed that the uterine tissue cultured in vitro for 4 days still expressed the ESCs marker (Vimentin) and endometrial epithelial cells (EPCs) marker (cytokeratin) (Fig. 4B). Treatment with rhIL-37b significantly increased the mRNA expression of Tgfb1 and decreased the mRNA expression of Mmp9, Vegfa, Il1b and Il10 (Fig. 4C), which is similar to ectopic lesions at 3 days after induction (Supplementary Fig. S5). In ectopic lesions at d3, the expression of Tgfb1 was notably increased, while the expression of Il1b, Il6, Tnfa, Il10, Mmp2, Mmp9, Vegfa and Il18r1 was decreased when compared with control (Supplementary Fig. S5A). Results for peritoneal fluid at d3 also documented reduced levels of TNF-α and MMP9 protein in the rhIL-37b treated group (Supplementary Fig. S5B).

Recent studies have revealed that Akt and the Erk1/2 signaling pathway were involved in the proliferation, adhesion, invasion and angiogenesis of endometrial cells (Chen et al., 2017; Lee et al., 2016; Li et al., 2012; Li et al., 2013; Xu et al., 2015). Therefore, we next measured the protein levels of Akt, p-Akt, Erk1/2 and p-Erk1/2 in uterine segments after rhIL-37b treatment by western blotting. The results showed that levels of p-Akt and p-Erk1/2 were reduced in segments treated with rhIL-37b (100 ng/mL) (Fig. 4D).

Effect of IL-37b on the proliferation, invasion and mRNA expression of endometrial cells

From the histological results we can see that ectopic lesions are composed mainly of ESCs and EPCs, so we next explored the effect of IL-37b on ESCs and EPCs. Isolated ESCs mostly expressed the ESCs marker Vimentin and showed little expression of the EPCs marker cytokeratin (Fig. 5A). ESC treated with rhIL-37b (i.e. exogenous IL-37b) showed significantly suppressed proliferation and invasion (Fig. 5B-C), decreased expression of Mmp9, Vegfa, Il1b, Il6, Tnfa, Il10, Il18r1 mRNAs and increased the expression of Tgfb1 and Sigirr mRNAs (Fig. 5D). We also investigated the effect of endogenous IL-37b on ESCs using a transfection assay with the pIL-37b plasmid, with pcDNA3.1 plasmid serving as the control. Real-time PCR and western blot results confirmed that the ESCs transfected with IL-37b plasmid expressed the IL-37b mRNA and protein (Fig. 6A-B). Transfection with IL-37b plasmid also suppressed ESCs proliferation and invasion (Fig. 6C-D), decreased the expression of Mmp2, Mmp9, Vegfa, Il1b, Il6, Tnfa, Il10, Il18r1 mRNAs and increased the expression of Tgfb1 and Sigirr mRNAs (Fig. 6E). In addition, we measured the levels of Akt, p-Akt, Erk1/2 and p-Erk1/2 protein in transfected cells and showed that cells transfected with IL-37b plasmid downregulated the levels of p-Akt and p-Erk1/2 (Fig. 6F).

As EPCs isolated from mouse uteri cannot proliferate in vitro, we used the cell line RL95–2 as a replacement. To the best of our knowledge, there is no report to date on the effect of IL-37b on IL-37 transgenic mice were protected from a variety of diseases, including colitis (McNamee et al., 2011), endotoxemia (Nold-Petry et al., 2015), spinal cord injury (Coll-Miro et al., 2016) and cerebral ischemia-reperfusion (Zhang et al., 2019). In this study, our results suggest that IL-37b inhibits endometriosis-like lesion growth, and endometrial cell proliferation and invasion, as well as angiogenesis and inflammation.

A previous study using a suture model, in which uterine tissue fragments were sutured to the peritoneal wall, revealed that treatment with rhIL-37b significantly decreased the size and weight of lesions (Jiang et al., 2018). In this study, we injected uterine segments i.p. to imitate the development of endometriosis which did not interfere in the process of lesion formation because uterine segments adhered spontaneously. Our results showed that treatment with rhIL-37b significantly inhibited the development of endometriosis. Furthermore, to further explore the role of IL-37b on the different stage of endometriosis, we assessed the effect of IL-37b on lesion formation and growth suggesting that rhIL-37 inhibited the development of endometriosis by inhibiting lesion growth.

Macrophages, as innate immune cells, play an important role in endometriosis (Capobianco and Rovere-Querini, 2013). Current studies have proved that macrophages were involved in fibrogenesis (Duan et al., 2018), angiogenesis (Capobianco et al., 2011; Lin et al., 2006) and neurogenesis (Wu et al., 2017) of endometriosis, and promoted ESCs proliferation when cocultured with ESCs (Itch et al., 2013). We used C cladonate Liposomes to deplete macrophages in order to assess

Discussion

Although IL-37 does not exist in mice, rhIL-37 protein has been proved to be effective in a large number of animal experiments (Coll-Miro et al., 2016; Sakai et al., 2012; Wu et al., 2014; Yang et al., 2015b). Moreover, overexpressing IL-37 in murine cells by transfection also has been shown, for example, to suppress inflammation (McCurdy et al., 2017; Schauer et al., 2017; Xu et al., 2020; Yang et al., 2015b; Ye et al., 2015). IL-37 transgenic mice were protected from a variety of diseases, including colitis (McNamee et al., 2011), endotoxemia (Nold-Petry et al., 2015), spinal cord injury (Coll-Miro et al., 2016) and cerebral ischemia-reperfusion (Zhang et al., 2019). In this study, our results suggest that IL-37b inhibits endometriosis-like lesion growth, and endometrial cell proliferation and invasion, as well as angiogenesis and inflammation.

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Figure 4 Effect of rhIL-37b on murine uterine segments in vitro. Histological results for the uterine segments cultured in vitro for 24 and 48 hours are shown, scale bar: 200 μm (A). The endometrial stromal cell (ESC) marker vimentin and epithelial cell (EPC) marker cytokeratin were detected by IHC in the uterine segment cultured for 4 days, scale bar: 50 μm (B). After treatment with different concentrations of rhIL-37b in vitro, the expression of inflammatory factors, Mmps and Vegfa and IL-37 receptors in uterine segments was detected by real-time PCR (C). After treating with rhIL-37b (100 ng/ml), the protein levels of Akt, p-Akt, Erk1/2 and p-Erk1/2 in uterine segments were analysed by western blotting (see also Supplementary Information). The relative phosphorylation of p-Akt to Akt and p-Erk1/2 to Erk1/2 was calculated after densitometric analysis of their relative protein expression, normalized to GAPDH (D). All experiments were independently performed three times. Student’s t-test and one-way ANOVA. *P < 0.05 or **P < 0.01.

the role of macrophages in the rhIL-37b treated mice. Our work has demonstrated that rhIL-37b treatment significantly inhibited the lesion growth, but the difference between the rhIL-37b and Clod+ rhIL-37b groups was negligible, which suggested that the effect of IL-37b on lesion growth was not dependent on macrophages. This may be because IL-37 and macrophages play important roles in different stages of endometriosis.

In this study, macrophage depletion significantly reduced early lesion formation but did not affect lesion growth, which was contrary to the effects of rhIL-37b. In contrast, another group using liposomes containing clodronate to deplete macrophages at different days (at -d1 for lesion adherence and implantation analyzing and at d4 and d8 for lesion growth analyzing) showed that macrophage depletion did not affect the adherence and implantation of lesions, but suppressed the growth of lesions (Bacci et al., 2009). However, another study has shown that macrophage depletion did not affect lesion weight through injecting clodronate-containing liposomes at -d1, d0, d3, d8 and d12 and measured the weight of lesions at d14 when com-
Figure 5 Effects of rhIL-37b on proliferation, invasion and mRNA expression levels in murine ESCs. The ESCs were stained with vimentin, pan-cytokeratin or PBS by IHC (scale bar: 500 μm), with PBS serving as control (A). ESCs were treated with rhIL-37b at different concentrations for 24 hours. Cell proliferation was examined by the Cell Counting Kit-8 (CCK-8) assay (B). Invasion ability was examined by a transwell assay and cell numbers were counted in ten randomly selected fields at 200× magnification (C). Real-time PCR examined the mRNA expression of Mmp2, Mmp9, Vegfa, inflammatory factors and IL-37 receptors (D). All experiments were independently performed three times. One-way ANOVA. *P < 0.05 or **P < 0.01.

pared with the group without clodronate (Zhao et al., 2015). These different protocols and controls maybe the reason for the different results across these studies. In addition, recent research has proved that the early phase of endometriosis (< 72 hours) predominantly depends on the immune system and predicted that targeting the innate immune system would prevent lesion attachment (Burns et al., 2018).

Depleting innate immune cells at an early stage of endometriosis, for example neutrophils, did reduce the lesion formation (Takamura et al., 2016).

Our results also showed that rhIL-37b treatment significantly downregulated the expression of proliferation marker PCNA in ectopic lesions and reduced the proliferation ability in endometrial cells.
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Invasion and angiogenesis are also involved in the growth of lesions. Our experiments have demonstrated that rhIL-37b treatment significantly down-regulated the expression of Mmp9 and Vegfa in ectopic lesions, uterine segments and endometrial cells and suppressed the invasion ability of ESCs. Transfected ESCs with IL-37b plasmid also reduced the expression of Mmp9 and Vegfa mRNA and significantly suppressed ESCs invasion. Previous studies have revealed that MMP inhibitor significantly reduced the number of ectopic lesions (Bruner et al., 1997), and anti-VEGF-A agents reduced the growth of endometriotic lesions in an animal model (Hull et al., 2003; Ricci et al., 2011). In addition, surgical removal of endometriotic lesions resulted in reduced serum levels of VEGF-A in patients with endometriosis (Bourlev et al., 2010). These results suggested that the therapeutic effect of IL-37b on endometriosis may be through inhibiting endometrial cell proliferation, invasion and angiogenesis.

Pelvic inflammation, as a classic feature of endometriosis, promotes ectopic proliferation and endometrial tissue growth, while endometriotic lesions contribute to pelvic inflammation in endometriosis (de Ziegler et al., 2010). Surgical removal of endometriotic lesions was shown to significantly reduce serum levels of inflammatory factors in endometriosis patients, which suggested that ectopic endometriotic lesions contributed to the systemic inflammation in endometriosis (Monsanto et al., 2016) and IL-37 overexpression significantly suppressed the expression of IL-1β, IL-6, TNF-α and IL-10 in ESCs from ectopic endometrium from ovarian endometriosis patients (Jiang et al., 2018). The pro-inflammatory factors IL-1β, IL-6 and TNF-α, and anti-inflammatory factors IL-10 and TGF-β, which were expressed abnormally in endometriosis patients (Rizner, 2015; Riccio et al., 2018), were suggested to play a role in the process of ectopic lesion formation or growth (Cheong et al., 2002; Suen et al., 2018).

Figure 6 Effects of over-expressing IL-37b on proliferation, invasion, gene expression and signaling pathway of murine ESCs. ESCs transfected with the pIL-37b plasmid expressed IL-37b mRNA and protein, as detected by real-time PCR (A) and western blotting (see also Supplementary Information) (B), while ESCs transfected with pcDNA3.1 plasmid did not (A-B). Compared with transfected with pcDNA3.1, the proliferation and invasion ability were significantly weakened in the ESCs transfected with pIL-37b (C-D). Real-time PCR results showed the mRNA expression of Mmp2, Mmp9, Vegfa, inflammatory factors and IL-37 receptors for each group (E). Western blotting detected the protein levels of Akt, p-Akt, Erk1/2 and p-Erk1/2. The relative phosphorylation of p-Akt to Akt and p-Erk1/2 to Erk1/2 were calculated after densitometric analysis of their relative protein expression, normalized to GAPDH (F). All experiments were independently performed three times. Student’s t-test. *P < 0.05 or **P < 0.01.
In the present study, we mainly focused on these factors. Treatment with the anti-inflammatory factor rhIL-37b protein not only decreased the mRNA expression of these factors in endometriosis-like lesions but also reduced the level of TNF-α in peritoneal fluids in mice. In addition, rhIL-37b treatment and pIL-37b plasmid transfection both significantly reduced the mRNA expression of Il1b, Il6, Tnfα and Il10 in ESCs. Treatment with rhIL-37b also reduced the expression of IL1B, TNFA and IL10 mRNA in epithelial cells RL95–2. Animal experiments have demonstrated that TNF-α, IL-6 or IL-10 inhibitors significantly decreased the size of lesions (Barrier et al., 2004; Falconer et al., 2006; Suen et al., 2014; Taskin et al., 2016). In addition, the weight of lesions in TGF-β1 null mice was significantly reduced when compared with wild-type mice (Hull et al., 2012). Although increased in lesions at d3, in cultured uterine segments and in endometrial cells, the expression of Tgfβ1 mRNA was decreased in lesions at d14. Similarly, IL-37 treatment increased the expression of TGF-β1 in Treg cell in vitro (Wang et al., 2016) and decreased the expression of TGF-β1 in the rat model of inflammatory myopathy in vivo (Yan et al., 2018). Therefore, we speculate that the difference in expression of TGF-β1 in our study may be related to the time of IL-37 treatment, which will require further experimental evidence. The findings described above suggested that the therapeutic effect of IL-37b on endometriosis may be achieved by inhibiting the development of inflammation.

IL-18Rα and IL-1R8 are the receptors of IL-37, which are required for IL-37 to carry out its anti-inflammatory function (Lunding et al., 2015; Nold-Petry et al., 2015). IL-18Rα participates in both pro- and anti-inflammatory responses, and the generation of IL-17-producing T helper cells (Gutcher et al., 2006; Lewis and Dinarello, 2006; Nold-Petry et al., 2009), while IL-1R8 participates in anti-inflammatory Th17 and Th2 responses (Bulek et al., 2009; Gulen et al., 2010; Molgora et al., 2018). In patients with endometriosis, the expression of IL-18Rα in ectopic lesions was higher when compared with normal endometrium (Oku et al., 2004). In general, the mRNA and protein expression of IL-1R8 are reduced in inflammatory conditions (Molgora et al., 2016). In our study, rhIL-37b treatment decreased the expression of Il-18rα and increased the expression of Il-1r8, which may contribute to the anti-inflammatory effect.

Previous studies have revealed that the phosphorylation of Akt and Erk1/2 in endometriosis patients was higher than those without endometriosis (Cinar et al., 2009; Honda et al., 2008; McKinnon et al., 2016; Yin et al., 2012; Yotova et al., 2011; Zhang et al., 2010), and IL-37 inhibited the phosphorylation of Erk1/2 in aortic valve interstitial cells (Zeng et al., 2017) and reduced the levels of p-Akt in hepatocellular carcinoma (Li et al., 2017b), but activated Erk1/2 and Akt signaling in endothelial cells (Yang et al., 2015a). In our study, both rhIL-37b treatment and pIL-37b transfection inhibited the phosphorylation of Akt and Erk1/2. Previous studies have demonstrated that Akt inhibitor reduced the number and the activity of ectopic lesions in vivo and decreased endometriotic cell proliferation in vitro (Leconte et al., 2011; Kim et al., 2014). Abrogating the phosphorylation of Erk1/2 decreased the pathology scores of ectopic lesions, which suggested a control of the progression of endometriosis, and decreased endometriotic cell proliferation in either non-endometriosis patients or endometriosis patients in vitro (Ngo et al., 2010). However, another study showed that inhibiting Akt or the Erk1/2 pathway did not decrease lesion...
growth, but both inhibiting Akt and Erk1/2 decreased the total number and total volume of lesions in mice (Arosh and Banu, 2019). All the above suggested that the effect of IL-37b on endometriosis may be through inhibiting Akt and Erk1/2 signaling pathways. However, the relationship between Akt or Erk phosphorylation and the expression of inflammatory factors (IL-1β, IL-6, TNF-α, IL-10 and TGF-β) and angiogenic factor (MMP9, VEGF) in endometriosis is still unclear and requires further study.

In summary, this study demonstrated that IL-37b suppressed the growth of lesions by inhibiting proliferation, invasion, angiogenesis and inflammation through Akt and the Erk1/2 signaling pathway, which suggested that IL-37b may have therapeutic effects in patients with endometriosis. Further research on the side effects of IL-37b would be required before clinical application.

Supplementary Data
Supplementary data are available at Molecular Human Reproduction online.

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Authors’ roles
Guimei Zhang, Ting Xiong and Zuchua Feng designed the study. Yongpei He, Fang Guo, Zhenzhen Du, Yixian-Fan and Huanhuan Sun performed all the experiments and collected data. Yongpei He analyzed data and drafted manuscript. All authors took part in revising this manuscript and approved the final version for publication.

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Conflict of interest
The authors declare that they have no conflicts of interest.

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